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• VIRAL HEPATITIS •

Diagnostic value of platelet derived growth factor-BB, transforming growth factor-b_1, matrix metalloproteinase-1, and tissue inhibitor of matrix metalloproteinase-1 in serum and peripheral blood mononuclear cells for hepatic fibrosis

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Abstract

AIM: Noninvasive diagnosis of hepatic fibrosis has become the focus because of the limited biopsy, especially in the surveillance of treatment and in screening hepatic fibrosis. Recently, regulatory elements involved in liver fibrosis, such as platelet derived growth factor-BB (PDGF-BB), transforming growth factor-β_1 (TGF-β_1), matrix metalloproteinase-1 (MMP-1), and tissue inhibitor of matrix metalloproteinase-1 (TIMP-1), have been studied extensively. To determine whether these factors or enzymes could be used as the indices for the diagnosis of hepatic fibrosis, we investigated them by means of receiver operating characteristic (ROC) curve.

METHODS: Serum samples from sixty patients with chronic viral hepatitis B and twenty healthy blood donors were assayed to determine the level of PDGF-BB, TGF-β_1, MMP-1, and TIMP-1 with ELISA, and HA, PCIII, C-IV, and LN level with RIA. The message RNA (mRNA) expression of TIMP-1 and MMP-1 in peripheral blood mononuclear cells (PBMCs) was detected by RT-PCR and Northern blot hybridization. Liver biopsy was performed in all patients. The biopsy samples were histopathologically examined. The trial was double-blind controlled.

RESULTS: The serum level of PDGF-BB, TIMP-1, the ratio of TIMP-1 and MMP-1 (TIMP-1/MMP-1), mRNA expression of TIMP-1 (TIMP-1mRNA), and the ratio of TIMP-1mRNA and MMP-1mRNA (TIMP-1mRNA/MMP-1mRNA) in patients was significantly higher than those in the healthy blood donors (t=2.514-11.435, P=0.000-0.016). The serum level of PDGF-BB, TIMP-1, TIMP-1/MMP-1, and TIMP-1mRNA was positively correlated with fibrosis stage and inflammation grade (r=0.239-0.565, P=0.000-0.033), while the serum level of MMP-1 was negatively correlated with fibrosis stage and inflammation grade, and TIMP-1mRNA/MMP-1mRNA was positively correlated with inflammation grade. Through the analysis by ROC curve, serum PDGF-BB was the most valuable marker, and its sensitivity was the highest among the nine indices. The markers with the highest specificity were TIMP-1mRNA and TIMP-1mRNA/MMP-1mRNA in PBMCs. The area under the curve (AUC) of PDGF-BB, TIMP-1mRNA, TIMP-1mRNA/MMP-1mRNA, TIMP-1/MMP-1, HA, PCIII, TIMP-1, C-IV, and LN was 0.985, 0.876, 0.792, 0.748, 0.728, 0.727, 0.726, 0.583, and 0.463, respectively. The sensitivity and the specificity in the parallel test was 99.0 % and 95.0 % when serum PDGF-BB, TIMP-1mRNA and TIMP-1mRNA/MMP-1mRNA was detected simultaneously.

CONCLUSION: Serum level of PDGF-BB, TIMP-1mRNA, TIMP-1mRNA/MMP-1mRNA in PBMCs, and serum level of TIMP-1 and TIMP-1/MMP-1 can be used as the indices for the diagnosis of hepatic fibrosis, but the former three are more useful. The combination of serum PDGF-BB, TIMP-1mRNA and TIMP-1mRNA/MMP-1mRNA in PBMCs is even more efficient in screening liver fibrosis.

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INTRODUCTION

Fibrosis is the leading cause of morbidity and mortality in hepatic diseases. More attention has been paid to its mechanism, diagnosis and treatment. The proper and rapid treatment depends on the accurate and simple diagnosis. Noninvasive diagnosis of hepatic fibrosis has become the focus because of the limited biopsy, especially in the surveillance of treatment and in screening hepatic fibrosis. Recently, regulatory factors involved in the mechanism of liver fibrosis such as PDGF-BB, TGF-β_1, interstitial enzyme, MMP-1 and its inhibitor, TIMP-1, have been studied extensively[1-14]. To find out whether these factors or enzymes could be used as the indices for diagnosis of liver fibrosis, protein level and mRNA expression were studied in sixty patients with chronic viral hepatitis B and twenty healthy blood donors. At the same time, these markers were compared with liver biopsy results and the routine serum markers (HA, PCIII, C-IV and LN) to identify their values in clinical practice via ROC curve and the combination test.

MATERIALS AND METHODS

Subjects

During the Sixth National Conference on Infectious and Parasitic Diseases in 2000, the Protocol of Prevention and Treatment for Viral Hepatitis (abbreviated as "2000 Criteria")[15] was modified. According to the "2000 Criteria", 60 patients with typical presentations of chronic hepatitis were included. Among them, 54 were men with an average age of 34.9±8.1 years, 6 were women with an average age of 36.6±1.0 years. Twenty-eight and thirty-two patients showed moderate and severe

degree of the disease, respectively. The patients' histories were mainly collected from the First Affiliated Hospital, School of Medicine, Zhejiang University and several other hospitals in Zhejiang Province between July 1998 and September 1999. All were positive in HBV markers without other viral infections or disorders except liver disease. The diagnosis was made by liver biopsy according to the "2000 Criteria". The normal control group included 20 healthy blood donors selected according to the random number table.

Histology

Biopsy samples of the liver >1 cm in length were fixed in 10 % neutralized formaldehyde, embedded in paraffin and stained with hematoxylin and eosin. The reticulin and Masson trichrome techniques were used specially for staining fibrous tissue components. Histological assessment of the liver for the division of fibrosis stage and inflammation grade, expressed as S1 to S4 and G1 to G4, was performed according to the "2000 Criteria".

Determination of serum level of PDGF-BB, TGFb_1, TIMP-1, MMP-1, HA, PCIII, C-IV, and LN

Serum specimens were stored at -20 ℃. The serum level of PDGF-BB, TGF-β_1, TIMP-1 and MMP-1 was assayed by ELISA. The kits of PDGF-BB and TGF-β_1 were provided by the American Genzyme Corporation, the American AND Corporation, respectively. The kits of TIMP-1 and MMP-1 were provided by the American ChemCon Corporation. The serum level of HA, PCIII, C-IV and LN was determined by RIA. The kits of HA, C-IV and LN were provided by Shanghai Navy Medical Institute. The PCIII kit was provided by Chongqing Tumor Institute. Assays were done following the manufacturers' manual.

Determiantion of TIMP-1mRNA and MMP-1mRNA in PBMCs

Total RNA extraction PBMCs were separated by Ficoll (GiBco. Life Technologies Inc) and the total RNA was extracted by Trizol reagent (GiBco. Life Technologies Inc).

Northern blot hybridization Total RNA 20 μg was denatured and undergone electrophoresis with 1 % agarose containing 2.2 mol/L formaldehyde and was transferred onto nitrocellulose membrane, which was dried at 80 ℃ for two hours. The filters were prehybridized at 68 ℃ for 1 hour in the solution containing 6×standard saline citrate (SSC), 5×Denhardt' s solution, 0.5×sodium dodecyl sulfate (SDS), and 100 mg/L salmon sperm DNA. The denatured probes were added into the solution for hybridization at 68 ℃ overnight. The filters were washed for three times, and then autoradiographed at -70 ℃.

RT-PCR Total RNA 1 μg and primer Oligo (dT) were used for reverse transcription (Promega). 5 μl reverse transcription template was used for amplification through PCR. The primers are MMP-1: 5' CTTCAGTGGTGATGTTCAGC3', 5' CATCGATATGCTTCAACGTTC3', 412 bp, TIMP-1: 5' GGAGTCCAGCAGACCACCTTA3', 5' -TGGGACACAG GTGCATGCCCTGCT-3', 110 bp. The amplified sequence length of β-actin is 224 bp. The PCR products were through 1.5 % (w/v) agarose gel electrophoresis and analyzed by gel imaging system.

Statistical analysis

Results were expressed as mean ± standard deviation ($\bar{x}\pm s$). *t* test and Spearman rank-correlation test were used. The results were considered statistically significant at $P<0.05$. Evaluation of the diagnostic test was made via ROC curve.

RESULTS

Comparison of serum level of PDGF-BB, TGF-b_1, TIMP-1, MMP-1 and TIMP-1/MMP-1 between patients and healthy blood donors

The serum level of PDGF-BB, TIMP-1 and TIMP-1/MMP-1 in 60 patients was significantly higher than that in the normal control group with an increase of 2.52, 0.5 and 1.67 fold, respectively. However, there was no difference in the serum level of MMP-1 and TGF-β_1 between patients and normal controls (Table 1). The serum level of MMP-1 was further studied. A declining tendency along the increase of fibrosis stage, inflammation grade and severity of the hepatic disease was observed while a significant difference appeared only between the patients in S4, G4 or with severe diseases and blood donors (Table 2). Moreover, it was also different between patients in S4 and those in S2 (Table 2). As to the serum level of TGF-β_1, there was no difference between patients and normal controls, even no significant difference among patients with the increase of fibrosis stage, inflammation grade and severity of the disease (Table 2).

Table 2 Comparison of the serum level of MMP-1 and TGF-β_1 between patients at different fibrosis stages, inflammation grades, and with different severities of the disease and the healthy controls ($\bar{x}\pm s$)

Group (*n*)	MMP-1(μg/L)	TGF-β_1(μg/L)
Controls (20)	7.98±3.13	26.28±16.69
S2(16)	6.34±2.96	31.66±18.01
S3(31)	6.80±5.34	34.19±26.23
S4(13)	3.93±2.93[ac]	25.16±20.90
G2(2)	7.66±0.36	18.25±6.01
G3(28)	6.41±4.91	31.89±21.61
G4(30)	5.61±4.15[a]	32.14±25.25
Mild disease (28)	6.56±3.66	34.39±24.43
Severe disease (32)	5.61±5.05	29.08±21.97

There was a statistically significant difference as compared with the control group [a]$P<0.05$, and a statistically significant difference as compared with S2 group [c]$P<0.05$.

Comparison of TIMP-1mRNA, MMP-1mRNA and TIMP-1mRNA/MMP-1mRNA in PBMCs between patients and healthy controls

As shown in Table 3, TIMP-1mRNA and TIMP-1mRNA/MMP-1mRNA were significantly elevated in patients than those in healthy controls with an increase of 1.6 and 1.79 fold, respectively. However, no difference was found in MMP-1mRNA expression between patients and healthy controls.

Table 1 Comparison of the serum level of PDGF-BB, TGF-β1, TIMP-1, MMP-1 and TIMP-1/MMP-1 between patients and healthy blood donors ($\bar{x}\pm s$)

Group (*n*)	PDGF-BB (ng/L)	TGF-β_1(μg/L)	TIMP-1(μg/L)	MMP-1(μg/L)	TIMP-1/MMP-1
Patients	67.75±30.08[b]	31.41±23.22	258.87±77.75[a]	6.05±4.44	83.66±100.36[b]
Controls	19.85±10.28	26.28±16.69	205.80±35.66	7.98±3.13	31.95±20.03
t	11.435	0.912	2.646	-1.79	3.176
P	0.000	0.365	0.01	0.077	0.002

There was a statistically significant difference as compared with the control group [a]$P<0.05$, [b]$P<0.01$.

Table 3 Comparison of TIMP-1mRNA, MMP-1mRNA and TIMP-1mRNA / MMP-1mRNA in PBMCs between patients and healthy controls ($\bar{x}\pm s$)

Group (n)	TIMP-1mRNA	MMP-1mRNA	TIMP-1mRNA / MMP-1mRNA
Patients (60)	1.05±0.69[b]	0.39±0.36	4.77±3.78[a]
Controls (20)	0.40±0.19	0.24±0.11	1.71±0.89
t	2.934	0.741	2.514
P	0.005	0.462	0.016

There was a statistically significant difference compared with the control group [a]P<0.05, [b]P<0.01.

Table 4 Relationship between serum level of PDGF-BB, TGF-β1, TIMP-1, MMP-1 and TIMP-1 / MMP-1, TIMP-1mRNA, MMP-1mRNA and TIMP-1mRNA / MMP-1mRNA and fibrosis stage and inflammation grade

Index	Fibrosis stage r	P	Inflammation grade r	P
TIMP-1	0.239[a]	0.033	0.261[a]	0.019
MMP-1	-0.333[b]	0.003	-0.266[a]	0.017
TIMP-1 / MMP-1	0.405[b]	0.000	0.340[b]	0.002
PDGF-BB	0.565[b]	0.000	0.534[b]	0.000
TGF-β1	-0.041	0.718	0.039	0.733
TIMP-1mRNA	0.366[b]	0.009	0.391[a]	0.015
TIMP-1mRNA / MMP-1mRNA	0.340	0.071	0.497[b]	0.006
MMP-1mRNA	0.091	0.582	0.001	0.995

[a]P<0.05, [b]P<0.01, compared with r_s threshold value.

Table 5 ROC curve analysis of nine indices

Index	AUC	P	Cut-off point	Sensitivity (%)	Specificity (%)	YI
PDGF-BB	0.985	0.000	≥40.50 ng / L	90.0	95.0	0.850
TIMP-1mRNA	0.876	0.000	≥0.79	73.7	100	0.737
TIMP-1mRNA / MMP-1mRNA	0.792	0.005	≥3.20	65.8	100	0.658
TIMP-1 / MMP-1	0.748	0.001	≥34.69	70.0	75.0	0.450
HA	0.728	0.003	≥145.20 μg / L	62.0	87.5	0.497
PCIII	0.727	0.004	≥137.40 mg / L	59.2	81.2	0.404
TIMP-1	0.726	0.003	≥254.00 μg / L	46.7	95.0	0.417
CIV	0.583	0.287	≥74.20 mg / L	55.1	68.7	0.238
LN	0.463	0.636	≥156.65 μg / L	37.8	75.0	0.128

YI (Youden Index)=sensitivity+specificity-1.

Table 6 Parameters of the combined diagnosis in the parallel test

Combined indices	Sensitivity (%)	Specificity (%)
TIMP-1mRNA+TIMP-1mRNA / MMP-1mRNA+PDGF-BB	99.0	95.0
PDGF-BB+TIMP-1mRNA+HA	99.0	83.1
TIMP-1mRNA+TIMP-1mRNA / MMP-1mRNA+PDGF-BB+HA	99.6	83.1
PDGF-BB+TIMP-1mRNA / MMP-1mRNA+HA	98.7	83.1
PDGF-BB+TIMP-1mRNA	97.4	95.0
TIMP-1mRNA+TIMP-1mRNA / MMP-1mRNA+HA	97.0	87.5
PDGF-BB+TIMP-1mRNA / MMP-1mRNA	96.6	95.0
PDGF-BB+HA	96.0	83.1
TIMP-1mRNA+TIMP-1mRNA / MMP-1mRNA	91.0	100
TIMP-1mRNA+HA	90.0	87.5
TIMP-1mRNA / MMP-1mRNA+HA	87.0	87.5

Table 7 Parameters of the combined diagnosis in the serial test

Combined indices	Sensitivity (%)	Specificity (%)
PDGF-BB+TIMP-1mRNA	66.3	100
PDGF-BB+TIMP-1mRNA / MMP-1mRNA	59.2	100
PDGF-BB+HA	56.0	99.4
TIMP-1mRNA+TIMP-1mRNA / MMP-1mRNA	48.5	100
TIMP-1mRNA+HA	45.7	100
PDGF-BB+TIMP-1mRNA+HA	41.1	100
TIMP-1mRNA / MMP-1mRNA+HA	40.8	100
TIMP-1mRNA+TIMP-1mRNA / MMP-1mRNA+PDGF-BB	40.4	100
PDGF-BB+TIMP-1mRNA / MMP-1mRNA+HA	36.7	100
TIMP-1mRNA+TIMP-1mRNA / MMP-1mRNA+HA	30.1	100
TIMP-1mRNA+TIMP-1mRNA / MMP-1mRNA+PDGF-BB+HA	27.1	100

Relationship between serum level of PDGF-BB, TGF-b_1,TIMP-1, MMP-1 and TIMP-1/MMP-1, TIMP-1mRNA, MMP-1mRNA and TIMP-1mRNA/MMP-1mRNA in PBMCs and fibrosis stage and inflammation grade

The serum level of PDGF-BB, TIMP-1, TIMP-1/MMP-1 and TIMP-1mRNA in PBMCs was positively correlated with fibrosis stage and inflammation grade, while the serum level of PDGF-BB had a stronger correlation than the other three indices. The serum level of MMP-1 was negatively correlated with fibrosis stage and inflammation grade. TIMP-1mRNA/MMP-1mRNA was positively correlated with inflammation grade. However, the serum level of TGF-β_1 and MMP-1mRNA was correlated with neither fibrosis stage nor inflammation grade.

Diagnostic value of serum level of PDGF-BB, TIMP-1, TIMP-1/MMP-1, MMP-1, HA, PCIII, CIV, LN and TIMP-1mRNA and TIMP-1mRNA/MMP-1mRNA in PBMCs

Table 5 shows that serum level of PDGF-BB, TIMP-1, TIMP-1/MMP-1, HA and PCIII, and TIMP-1mRNA and TIMP-1mRNA/MMP-1mRNA in PBMCs could be used to diagnose hepatic fibrosis. Among them, the serum level of PDGF-BB was most useful for its AUC and YI were close to 1, followed by TIMP-1mRNA and TIMP-1mRNA/MMP-1mRNA in PBMCs. Furthermore, the serum level of PDGF-BB was most sensitive, and the next was TIMP-1mRNA and TIMP-1mRNA/MMP-1mRNA in PBMCs. TIMP-1mRNA and TIMP-1mRNA/MMP-1mRNA in PBMCs were most specific, followed by the serum level of PDGF-BB and TIMP-1. When both the sensitivity and the specificity were taken into consideration, serum PDGF-BB, serum HA, TIMP-1mRNA and TIMP-1mRNA/MMP-1mRNA were the relatively efficient indices.

Diagnostic value of the single index combination

When both the sensitivity and the specificity needed to be taken into account, we could combine the following four indices, namely serum PDGF-BB, serum HA, TIMP-1mRNA/MMP-1mRNA and TIMP-1mRNA in PBMCs. Table 6 indicates that the combination of serum level of PDGF-BB, TIMP-1mRNA and TIMP-1mRNA/MMP-1mRNA in PBMCs was more useful in the parallel test. However, no efficient combination in the serial test was observed (Table 7).

DISCUSSION

Hepatic fibrosis is characterized by imbalanced deposition and degradation of extracellular matrix (ECM). Many factors are involved in the process. Thus, it is difficult to evaluate the fibroproliferative activity. Liver biopsy is still regarded as the gold standard for the diagnosis of fibrosis, but even elaborated scores of histological activity are limited to evaluate the prognosis in the individual case. Moreover, it is not convenient, to some degree, to use it as a routine method for the diagnosis, evaluation and supervision of the disease in clinical practice. Unfortunately, there have been no established noninvasive markers or tests for the diagnosis of hepatic fibrosis. Therefore, it is essential to explore the noninvasive and reliable indices for assessing the progress of liver fibrosis. Previously, we revealed the diagnostic value of ultrasonography on assessing liver fibrosis resulted from Schistosomiasis japonica[16], but sometimes we could not distinguish benign fatty infiltration from fibrosis, because both of their echogenicities appeared increased or diffuse. Detection of the biochemical indices in serum has been the focus in the filed of hepatic fibrosis. At present, the serum indices revealing the progress of hepatic fibrosis mainly include two kinds, reflecting deposition and degradation. Previously, more attention was paid to the factors reflecting the deposition or metabolism of ECM, and the serum level of HA, PCIII, PIIIP, CIVand LNwas studied. Zheng *et al*[17,18] confirmed the clinical value of the serum fibrosis indices (HA, PCIII, CIVand LN) through comparing them with the histological reports performed on liver fibrosis patients resulted from chronic hepatitis B. Furthermore, the serum level of HA was considered to be the most sensitive among the four indices, this result was also reported by others[19,20]. However, the four indices did not fully reflect the histological changes and were often influenced by other factors, moreover, in some chronic hepatitis B cases, they did not correspond to the biopsy results[21]. Thus, it is important and necessary to explore new and more reliable indices. With the mechanism of hepatic fibrosis elucidated further, the focus has been the elements involved in the degradation of ECM such as MMP-1[14,22,23] and TIMP-1[24-27] and the regulatory factors such as PDGF-BB and TGF-β_1 whose vital roles in hepatic fibrosis have been confirmed.

To establish the noninvasive index, the first step was to observe whether it demonstrated a difference between patients and normal controls. In our study, we compared the serum level of PDGF-BB, TIMP-1, MMP-1, TIMP-1/MMP-1 and TGF-β_1 between 60 fibrosis patients and 20 healthy blood donors. The serum level of PDGF-BB, TIMP-1 and TIMP-1/MMP-1 in patients was significantly elevated than that in healthy controls. However, the serum level of MMP-1 demonstrated a declining tendency with the severity of liver fibrosis, inflammation and the disease condition although the difference between two groups existed only when the patients were in S4, G4 or with severe hepatitis. This result indicted that the abnormal serum MMP-1 did not appear until the patients were in the advanced fibrosis. With regard to TGF-β_1 serum level, it was not different from that of the control group with the progress of liver fibrosis, inflammation and the severity of the disease, showing that serum TGF-β_1 may not be sensitive as a diagnostic index. Similar results were also reported. Daniluk *et al*[28] found that serum level of TGF-β_1 in alcohol-related liver cirrhosis was similar to that in controls. Oberti *et al*[29] detected several indices of chronic hepatitis patients, including HA, PT, GGT, alph2 macroglobulin, PIIIP, LN and TGF-β_1. They found that HA and PT were significant in cirrhosis. In fact, TGF-β_1 is secreted from cells in the manner of the complex formed by TGF-β_1 and its binding protein. However, pre TGF-β_1 can be activated to its active form only after its binding protein is released. This does not mean that TGF-β_1 may play a role freely once it is released into blood, for its corresponding receptors still block it. There are a lot of studies about the correlation of plasma TGF-β_1 with chronic hepatitis, liver fibrosis or cirrhosis[30,31]. But, in general, analysis of plasma level is fraught with difficulties related to contamination of the sample by TGF-β from platelets. Moreover, the plasmin in the plasma may increase the amount of TGF-β_1 through opening the LAP-TGF-β_1 complex. Clearance of TGF-β is also complicated. It binds locally at sites of injury to ECM and generally to vascular endothelium, it may be sequestered by soluble proteins, and can also undergo renal excretion or be taken up by hepatocytes. These modes of sequestration or clearance may vary at different circumstances. Thus, increase in plasma TGF-β may not reflect pericellular concentrations at the injury site, and due to this reason, plasma TGF-β is unlikely to be diagnostically useful[32,35]. But Kobayashi *et al*[36] found that serum TGF-β_1 could be used as an accurate indicator of progressive fibrogenesis in postoperative biliary atresia patients. The reason for the disagreement may be the different criteria for the division of liver fibrosis.

Correlation analysis was carried out to elucidate the cause leading to the difference in some indices between patients and normal controls. We found that the serum level of PDGF-BB,

TIMP-1 and TIMP-1/MMP-1 was positively correlated with fibrosis stage and inflammation grade while the serum level of MMP-1 was inversely correlated with fibrosis stage and inflammation grade. The data indicated that histological changes could directly result in the higher level of serum PDGF-BB, serum TIMP-1 and TIMP-1/MMP-1 in patients and could explain the declining tendency appearing in the comparison between each group of patients and the controls as well. Serum level of TGF-β_1 was not correlated with fibrosis stage or inflammation grade. This may confirm that detection of serum TGF-β_1 was not reliable in clinical practice.

It has been reported that mRNA levels of TIMP and MMP and corresponding proteins were related to liver fibrosis or cirrhosis. Chen *et al*[37] studied the collagen metabolism of liver fibrosis at transcription level in rabbits infected by Schistosomiasis japonica, and found that mRNA levels of MMP-1 and MMP-9 declined almost to the normal level at the later stage of fibrosis. Yata *et al*[38] found that mRNA expression of hepatic TIMP-1 increased in hepatic fibrosis. Lichtinghagen *et al*[27] investigated the mRNA levels of hepatic TIMP-1, 2, 3 and MMP-2, 7, 9 in 29 chronic active hepatitis C patients (CAH) and 7 cirrhosis patients resulted from hepatitis C virus, and found that none of mRNA levels was significantly different between CAH patients with and without fibrosis, while MMP-2, MMP-7, and TIMP-1 provided the best discrimination between cirrhosis and pre-cirrhotic stages. Lichtinghagen *et al*[39] found that mRNA expression of MMP-2, MMP-9 and TIMP in peripheral blood cells had no correlation with the circulating concentrations of these proteins, which indicated that detection of MMP mRNA and TIMP mRNA in peripheral blood cells may also give us important information about liver fibrosis. Boker *et al*[40] reported that TIMP-1 could be detected in lymphocytes and granulocytes. To determine whether TIMP-1mRNA and MMP-1mRNA in PBMCs could be used as the diagnostic markers, we detected them and TIMP-1mRNA/MMP-1mRNA, and compared these indices between patients and the healthy blood donators. The results demonstrated that TIMP-1mRNA, TIMP-1mRNA/MMP-1mRNA significantly increased while no change in mRNA expression of MMP-1 was observed. Correlation analysis revealed that TIMP-1mRNA was positively correlated with fibrosis stage and inflammation grade, while TIMP-1mRNA/MMP-1mRNA was only positively correlated with inflammation grade. No relationship was found between MMP-1mRNA and fibrosis stage or inflammation grade. With regard to MMP-1mRNA in PBMCs, no statistical difference may attribute to the higher standard deviation among individual values. Other factors influencing mRNA expression may also involve. Therefore, it could be more useful to detect TIMP-1mRNA in PBMCs for evaluating liver fibrosis.

As there was a difference in the serum level of PDGF-BB, TIMP-1, TIMP-1/MMP-1, MMP-1, TIMP-1mRNA and TIMP-1mRNA/MMP-1mRNA in PBMCs between patients and the normal controls, we wanted to know whether these indices could be used for diagnosis, and if they could, the following problem was whether they were more valuable than those four routine serum markers (HA, PCIII, CIV, LN). AUC and YI of the serum level of PDGF-BB were the closest to 1 through ROC curve. This revealed that the diagnostic value of serum level of PDGF-BB was the highest among nine indices (Table 5). Although TGF-β_1 as the main fibrogenic mediator mediates HSC activation and transformation, additional growth factors like PDGF become important in the later stage of HSC transformation. That means PDGF is vital in the progress of liver fibrosis. PDGF has been proved to be the main stimulator of HSC proliferation, migration and the strong mitogen for HSCs. Among the three subunits-AA, AB and BB, PDGF-BB is the vital cytokine for the signaling pathway in HSC and other cells[41-44]. In recent years, studies have not been adequately performed on the serum level of PDGF-BB for assessing liver fibrosis. Our results indicated that detection of the serum level of PDGF-BB had profound significance. TIMP-1mRNA and TIMP-1mRNA/MMP-1mRNA in PBMCs was inferior to serum PDGF-BB. The diagnostic value of serum level of HA, PCIII, TIMP-1 and TIMP-1/MMP-1 is similar, for their AUC are closer, but detection of serum level of HA and TIMP-1/MMP-1 was more applicable if we evaluated them according to YI. Researchers have studied TIMPs from hepatic tissues, serum level to mRNA expression, revealing the important relationship of TIMPs with fibrosis stage or inflammation grade. But it is still unclear whether the serum level of TIMP-1 and TIMP-1mRNA in PBMCs can be used as the markers for the diagnosis of liver fibrosis. If they can, are they superior or inferior to other established markers[45-47]? Our results demonstrated that TIMP-1mRNA was more sensitive than TIMP-1. Some studies[26,39] have revealed the role of the ratio of MMPs and TIMPs such as MMP-1/TIMP-1, MMP-2/TIMP-1, but did not report whether the ratio could be used for the diagnosis of liver fibrosis. We observed that both TIMP-1mRNA/MMP-1mRNA in PBMCs and TIMP-1/MMP-1 in serum could be used for the diagnosis of hepatic fibrosis. However, the former was superior to the latter. The data suggested that we should take both the protein level and mRNA expression into account to explore the noninvasive markers. The value of the serum level of CIV and LN was relatively low. In fact, their AUC values were 0.5 for their *P* values were above 0.05. The roles of serum MMPs in liver fibrosis had been studied[22,47]. Murawaki *et al*[22] found that the serum MMP-1 test was superior to the serum PIIINP test in assessing liver necroinflammation, and thought that the serum MMP-1 test might be useful clinically to differentiate active from inactive types of hepatitis in patients with chronic viral hepatitis, but they did not elucidate whether serum MMP-1 could be more efficient than other indices for assessing liver fibrosis. We evaluated the diagnostic value of MMP-1 for pre-cirrhosis and severe inflammation as there was a difference between patients in S4 or G4 and the normal controls. But the results revealed that the serum level of MMP-1 was of no use for assessing liver fibrosis and evaluating the severity of inflammation. However, our results revealed the declining tendency of the serum MMP-1 with the progress of hepatic fibrosis. The problem is worth further investigating.

The diagnostic value, the sensitivity and the specificity should be taken together, when an index is evaluated for the diagnosis of liver fibrosis. ROC curve analysis revealed that the sensitivity and the specificity of one index were not desirable. Thus, to overcome the limitation of the single index, combination test should be used. There are two kinds of combination test in clinical practice. One is the parallel test and the other is the serial test. The former is often used to screening diseases because it focuses on improving the sensitivity and decreasing the missing incidence. The latter is used to confirm the diagnosis. Table 6 showed that indices in the parallel test were more sensitive than one index. Furthermore, its specificity was also improved. These results revealed that the parallel test was beneficial to screening hepatic fibrosis because hepatic fibrosis continue to progress even though the pathogen have been eliminated. Table 7 showed that the specificity was improved, while the sensitivity was evidently decreased. The data demonstrated that the combination of serum PDGF-BB, HA, TIMP-1mRNA, and TIMP-1mRNA/MMP-1mRNA in PBMCs was clinically limited. However, the specificity of each kind of the combination in the serial test reached close to 100 %, indicating that the diagnostic value of any kind of combination was important and could provide the key information for doctors once some abnormal

results appeared.

In conclusion, we think that serum PDGF-BB, TIMP-1, TIMP-1/MMP-1, TIMP-1mRNA and TIMP-1mRNA/MMP-1mRNA in PBMCs may be used to diagnose hepatic fibrosis. Among them, serum PDGF-BB, TIMP-1mRNA and TIMP-1mRNA/MMP-1mRNA are more sensitive and could be used in clinical practice. The combination of serum PDGF-BB, TIMP-1mRNA and TIMP-1mRNA/MMP-1mRNA in PBMCs is more efficient in screening liver fibrosis. However, the ideal combination for confirming the diagnosis need to be further explored.

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